Atorvastatin Upregulates Type III Nitric Oxide Synthase in Thrombocytes, Decreases Platelet Activation, and Protects From Cerebral Ischemia in Normocholesterolemic Mice

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Background and Purpose—Thrombosis superimposed on atherosclerosis causes approximately two thirds of all brain infarctions. We previously demonstrated that statins protect from cerebral ischemia by upregulation of endothelial type III nitric oxide synthase (eNOS), but the downstream mechanisms have not been determined. Therefore, we investigated whether antithrombotic effects contribute to stroke protection by statins.

Methods—129/SV wild-type and eNOS knockout mice were treated with atorvastatin for 14 days (0.5, 1, and 10 mg/kg). eNOS mRNA from aortas and platelets was measured by reverse-transcriptase polymerase chain reaction. Platelet factor 4 (PF 4) and β-thromboglobulin (β-TG) in the plasma were quantified by ELISA. Transient cerebral ischemia was induced by filamentous occlusion of the middle cerebral artery followed by reperfusion.

Results—Stroke volume after 1-hour middle cerebral artery occlusion/23-hour reperfusion was significantly reduced by 38% in atorvastatin-treated animals (10 mg/kg) compared with controls. Serum cholesterol levels were not affected by the treatment. eNOS mRNA was significantly upregulated in a dose-dependent manner in aortas and in thrombocytes of statin-treated mice compared with controls. Moreover, indices of platelet activation in vivo, ie, plasma levels of PF 4 and β-TG, were dose-dependently downregulated in the treatment group. Surprisingly, atorvastatin-treatment did not influence PF 4 and β-TG levels in eNOS knockout mice.

Conclusions—The synthetic 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor atorvastatin upregulates eNOS in thrombocytes, decreases platelet activation in vivo, and protects from cerebral ischemia in normocholesterolemic mice. Antithrombotic and stroke-protective effects of statins are mediated in part by eNOS upregulation. Our results suggest that statins may provide a novel prophylactic treatment strategy independent of serum cholesterol levels. (Stroke. 2000;31:2437-2449.)

Key Words: blood platelets ▪ cerebral ischemia ▪ HMG-CoA reductase inhibitors ▪ nitric oxide ▪ thrombosis

A growing body of evidence suggests that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, exert beneficial vascular effects that are independent of their cholesterol-lowering potencies.1–6 Thus far, the relevance of cholesterol as a stroke risk factor has remained controversial,7,8 and several intervention trials with non-statin lipid-lowering drugs failed to demonstrate a reduction in stroke incidence.9,10 Recent trials demonstrated that statins lower the risk of stroke up to 42% in patients with coronary heart disease.11 Surprisingly, statins significantly reduced cardiovascular events even in patients with average cholesterol levels.12–14 It has been suggested that the protective mechanism(s) may relate to improved endothelial function and/or antithrombotic effects,1–6 but the mechanism of the cholesterol-independent effects of statins is not clear.

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Upregulation of endothelial type III nitric oxide synthase (eNOS) was recently identified as a novel mechanism of action of statins in vitro and in vivo.15–19 Endothelium-derived NO has been shown to regulate blood pressure, augment regional blood flow, improve cerebral circulation, decrease leukocyte activation, and inhibit platelet aggregation.20–24 Accordingly, animals lacking eNOS expression (eNOS knockout mice) suffer from arterial hypertension, develop enlarged cerebral infarcts after occlusion of the middle cerebral artery, and have enhanced hemostasis.20,24,25 Indeed, eNOS upregulation by statins augments cerebral blood flow in mice as a potential mechanism of stroke protection.15,26 Since thrombosis superimposed on atherosclerosis causes approximately two thirds of all brain infarc-
tions,\textsuperscript{27} we investigated whether antithrombotic effects contribute to the protective effects of statins in vivo.

\(\beta\)-Thromboglobulin (\(\beta\)-TG) and platelet factor 4 (PF 4) are 2 platelet-specific proteins that are secreted from the \(\alpha\)-granules during the release reaction induced by ADP, epinephrine, arachidonic acid, collagen, and thrombin.\textsuperscript{28} Plasma levels of these factors are established and valid indices of platelet activation in vivo.\textsuperscript{28} Hence, we investigated whether increased eNOS activity induced by statins would regulate markers of platelet activation. Moreover, we hypothesized that the source of increased NO production after statin treatment is not only the endothelium but also the blood platelets themselves. In fact, active type III NOS has recently been identified in human platelets,\textsuperscript{29} and NO released from activated platelets inhibits platelet recruitment to a growing thrombus.\textsuperscript{30}

Materials and Methods

Drugs

Atorvastatin was a gift from Gödecke AG Freiburg. Stock solutions (2 mg/mL) were prepared in PBS and 5% (vol/vol) ethanol (pH 7.6).

Animals and Drug Treatment

Animal experiments were conducted in strict accordance with national and institutional guidelines. 129/SV wild-type (weight, 18 to 22 g) or eNOS knockout mice\textsuperscript{30} were treated with atorvastatin (0.5, 1, and 10 mg/kg) or a corresponding vehicle of daily subcutaneous injections for 14 days. Serum cholesterol levels were determined by the Institut für Klinische Chemie, Universität zu Köln, Germany.

Ischemia Model

Animals were anesthetized for induction with 1.5% halothane and maintained in 1.0% halothane in 70% N\textsubscript{2}O and 30% O\textsubscript{2} with a vaporizer. Ischemia experiments were essentially performed as described.\textsuperscript{15,31} In brief, brain ischemia was induced with an 8.0-nylon monofilament coated with a silicone resin/hardener mixture (Xantopren M Mucosa and Activator NF Optosil Xantopren, Haereus Kulzer) as described.\textsuperscript{15,31} The filament was introduced into the left median line. After a careful dissection, a tight ligature (with a diameter of 0.05 mm) was placed around the inferior vena cava, just below the left renal vein. Two hours later, the abdomen was reopened under anesthesia. The thrombus, if present, was removed, washed in distilled water, blotted on filter paper, and placed in a desiccator; 24 hours later, the dry weight of the thrombus was recorded.

Preparation of Platelet-Poor Plasma and Platelet-Rich Plasma

Animals were deeply anesthetized with 0.1 mL chloral hydrate (7% wt/vol in PBS) by intraperitoneal injection. Whole blood was withdrawn by puncture of the retro-orbital plexus. For PF 4 and \(\beta\)-TG measurements, anticoagulation was performed with modified Edinburgh anticoagulant (10% vol/vol; Hemogard CTAD, Diatube H, Becton Dickinson, Diagnostica Stago, reference 367599). After 15 minutes of incubation at 37°C, blood was collected at 2000 rpm for 30 minutes (4°C). Only the medium phase of the supernatant (platelet-poor plasma) was withdrawn with a pipette and stored at −70°C until further use.

For preparation of platelet-rich plasma (PRP), blood was immediately anticoagulated with trisodium citrate (10% vol/vol of a solution containing 130 mmol/L citric acid, 125 mmol/L trisodium citrate, and 110 mmol/L glucose). Blood was centrifuged (150g, 6 minutes, 22°C), and the supernatant, which represents PRP, was separated. Counting of platelets and white (WBC) and red blood cells (RBC) in PRP was performed with a CellDyn3500 analyzer (Abbott; auxiliary mode). The concentration of both WBC and RBC in PRP was negligible (WBC, 0.004 ± 0.001 × 10\textsuperscript{6}/μL; RBC, 0.007 ± 0.002 × 10\textsuperscript{6}/μL; platelets, 110 ± 23 × 10\textsuperscript{6}/μL). Additionally, platelets were examined under phase contrast microscopy to check for contamination of other blood cells; a few RBC but virtually no WBC were detected.

Measurement of Plasma Levels of PF 4 and \(\beta\)-TG

PF 4 was quantified in CTAD plasma with the use of the Asserachrom PF4 ELISA from Roche, Diagnostica Stago (reference 1875 353). \(\beta\)-TG was quantified in CTAD plasma with the use of the Asserachrom \(\beta\)-TG ELISA from Roche, Diagnostica Stago (reference 1875 370). The assay and calculation of results were performed according to the manufacturer’s instructions.

Reverse-Transcriptase Polymerase Chain Reaction

Aortas were quickly frozen after the animals were killed. Isolated platelets from PRP were dissolved in RNA-clean (AGS) and stored at −70°C until RNA preparation. Total RNA isolation, reverse transcription, and competitive polymerase chain reaction (PCR) was performed according to standard techniques.\textsuperscript{16} The sense (5’- TCTCGCCTGCCA CCTGTACCTCCTAA-3’) and antisense (5’- AACATATGTC TTGCTCAAGGCA-3’) primers were used to amplify a 340-bp murine eNOS cDNA fragment and a 1052-bp mutated eNOS cDNA that served as internal standard. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as an external standard as described.\textsuperscript{17} Each PCR cycle consisted of denaturing at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 60 seconds. The linear exponential phases for eNOS and GAPDH PCR were 35 and 25 cycles, respectively. Equal amounts of corresponding NOS and GAPDH reverse transcriptase PCR (RT-PCR) products were loaded on 1.5% agarose gels, and optical densities of ethidium-bromide-stained DNA bands were quantified and expressed as mean ± SEM of the ratio of murine eNOS to eNOS mutant PCR signal.

Rho GTP-Binding Assay

The Rho GTP-binding activity was determined by immunoprecipitation of \([\text{32P}]\text{GTP}\)-S-labeled Rho.\textsuperscript{17} Aortas were quickly isolated and snap-frozen in isopentane on dry ice. Briefly, membrane and cytosolic proteins were isolated and incubated (20 μg) for 30 minutes at 37°C in a buffer containing \([\text{32P}]\text{GTP}\-S (20 mmol/L), GTP (2 μmol/L), MgCl\textsubscript{2} (5 mmol/L), EGTA (0.1 mmol/L), NaCl (50 mmol/L), creatinine phosphate (4 mmol/L), phosphatecitric acid (5 U), ATP (0.1 mmol/L), dithiothreitol (1 mmol/L), leupeptin (100 μg/mL), aprotinin (50 μg/mL), and phenylmethylsulfonyl.
fluoride (2 mmol/L). The assay was terminated with excess unla-
beled GTP<sup>g</sup><sub>S</sub> (100 μmol/L). Samples were then resuspended in 100 μL of immunoprecipitation buffer containing Triton-X (1%), SDS (0.1%), NaCl (150 mmol/L), EDTA (5 mmol/L), Tris-HCl (25 mmol/L, pH 7.4), leupeptin (10 μg/mL), aprotinin (10 μg/mL), and phenylmethylsulfonyl fluoride (2 mmol/L). The RhoA antiserum was added to the mixture at a final dilution of 1:75. The samples were allowed to incubate for 16 hours at 4°C with gentle mixing. The antibody-G-protein complexes were then incubated with 50 μL of protein A-Sepharose (1 mg/mL, Pharmacia Biotech Inc) for 2 hours at 4°C, and the immunoprecipitate was collected by centrifugation at 12,000 g for 10 minutes. The pellets were washed 4 times in a buffer containing HEPES (50 mmol/L, pH 7.4), NaF (100 μmol/L), sodium phosphate (50 mmol/L), NaCl (100 μmol/L), Triton X-100 (1%), and SDS (0.1%). The final pellet containing the immunoprecipitated [35 S]GTP<sup>g</sup><sub>S</sub>-labeled Rho proteins was counted in a liquid scintillation counter (LS 1800, Beckman Instruments, Inc). Nonspecific activity was determined in the presence of excess unlabeled GTP<sup>g</sup><sub>S</sub> (100 μmol/L).

Data Analysis
Data are presented as mean±SEM. Comparisons were made by 2-tailed Student’s t test and ANOVA. P<0.05 was considered statistically significant.

Results
Atorvastatin Protects From Cerebral Ischemia/Reperfusion
To evaluate whether chronic pretreatment with the synthetic statin atorvastatin confers protection after stroke, 129/SV wild-type mice were injected daily with atorvastatin (10 mg/kg SC) for 14 days. Under these treatment conditions, serum cholesterol levels were not significantly altered (87.8±15.4 versus 89.5±8.5 mg/dL in atorvastatin- versus vehicle-treated mice, respectively; n=4 animals). Animals were subjected to 1-hour middle cerebral artery occlusion followed by 23 hours of reperfusion. Lesion volume was determined after 24 hours on 2,3,5-triphenyltetrazolium–stained 2-mm coronal brain sections by computer-assisted volumetry. Direct lesion volume was reduced by 38% in atorvastatin-treated animals (Figure 1a). Significantly smaller lesions were evident in 2 (ie, sections 2 and 3) of the 5 standardized coronal brain sections (Figure 1b). When infarction volume was corrected for brain swelling (ie, calculated with the indirect method), infarcts in the atorvastatin-treated group were still significantly decreased by 35% (50.2±7.8 versus 77.6±10.8 mm<sup>3</sup> in atorvastatin- versus vehicle-treated mice, respectively; n=12 and 9 animals; P<0.05).

Atorvastatin Upregulates eNOS mRNA Expression in Aortas
To examine whether treatment with atorvastatin regulates eNOS expression in the vasculature, eNOS mRNA levels were determined in the aorta by RT-PCR (Figure 2a). Treatment with atorvastatin (0.5, 1, and 10 mg/kg SC) daily for 14 days compared with vehicle-injected animals. A 1052-bp mutated eNOS cDNA (Mutant) served as internal standard and GAPDH as external standard. b, Treatment with atorvastatin increased eNOS mRNA ratio by 1.1- to 2.3-fold. Data are presented as mean and SEM; n=4 to 8 animals. *P<0.05.

Atorvastatin Inhibits Rho GTPase Activity in Aortas
We previously demonstrated in vitro that the mechanism by which statins increase eNOS expression relates to the inhibition of geranylgeranylation of the small GTP-binding protein...
However, it is not known whether statin treatment indeed inhibits Rho function in the vessel wall. Hence, to investigate whether atorvastatin would decrease Rho GTPase activity in vivo, we performed GTP-binding assays in mouse aortas after atorvastatin pretreatment. In fact, Rho GTPase activity was significantly inhibited by atorvastatin pretreatment (10 mg/kg for 14 days) (Figure 3). In accordance with the in vitro evidence, these results suggest that atorvastatin upregulates eNOS expression by inhibition of Rho isoprenylation.

Atorvastatin Upregulates eNOS mRNA in Platelets In Vivo

NO is a mediator of platelet function and is released from platelets in vivo. Therefore, we determined whether platelets from 129/SV wild-type mice treated with atorvastatin for 14 days contain higher levels of eNOS mRNA (Figure 4a). Indeed, RT-PCR analysis revealed that treatment with atorvastatin (0.5, 1, and 10 mg/kg) significantly increased the eNOS in platelets by 1.2-, 1.8-, and 3.2-fold, respectively (n=4 to 8 animals; *P<0.05 for 10 mg/kg versus control) (Figure 4b).

Atorvastatin Downregulates Indices of Platelet Activation In Vivo and Inhibits Thrombus Formation

To determine whether the increased expression of type III NOS in the aorta and platelets after statin treatment have an effect on platelet function, 2 markers of platelet activity, ie, PF 4 and β-TG, were quantified in plasma by means of ELISA. Compared with vehicle, we found that PF 4 was decreased by 15%, 24%, and 57% after treatment (14 days) with atorvastatin at 0.5, 1.0, and 10 mg/kg, respectively (17.8±0.3 versus 15.1±0.7 versus 13.5±7.7 versus 7.7±1.0 IU/mL in vehicle-treated versus 0.5, 1, and 10 mg/kg atorvastatin-treated mice, respectively; n=4 to 10 animals; *P<0.05 for 1 and 10 mg/kg versus control) (Figure 5a).

Similarly, atorvastatin treatment reduced the plasma levels of β-TG compared with control by 8%, 20%, and 31% (2.6±0.2 versus 2.4±0.4 versus 2.1±0.3 versus 1.6±0.2 IU/mL in vehicle-treated versus atorvastatin-treated mice, respectively; n=4 to 10 animals; *P<0.05 for 1 and 10 mg/kg versus control) (Figure 5b).

Additionally, we subjected mice to experimental thrombosis after treatment with atorvastatin (10 mg/kg for 14 days) or vehicle. While thrombus formation was observed in all vehicle-injected mice (9/9 mice; thrombus weight=1.8±0.4 mg), thrombus formation was observed in only 25% of atorvastatin-treated mice (2/8 mice; thrombus weight=1.1±0.2 mg).
No Antithrombotic Effects of Statins in eNOS Knockout Mice

To test whether the regulation of platelet function by atorvastatin treatment was indeed mediated by NO produced by type III NOS, eNOS knockout (eNOS−/−) mice were subjected to the same treatment protocol with atorvastatin (10 mg/kg, 14 days). Surprisingly, atorvastatin did not alter plasma levels of PF 4 (Figure 6a) (n=4 animals) or the levels of β-TG (Figure 6b) (n=4 animals). These findings suggest that most, if not all, effects of atorvastatin on platelet activity were mediated by eNOS.

Discussion

This study shows that treatment with the synthetic HMG-CoA reductase inhibitor atorvastatin decreases platelet activation and protects wild-type mice from cerebral ischemia. Atorvastatin treatment did not significantly alter cholesterol levels. Thus, reduction of platelet activation in our model is unrelated to the lipid-lowering properties of statins. Surprisingly, atorvastatin had no effect on platelet activation in eNOS knockout mice, demonstrating that the underlying mechanism is indeed the upregulation of type III NOS by statin treatment.

These findings extend our previous data showing stroke protection in mice with simvastatin pretreatment, demonstrating a class effect for HMG-CoA reductase inhibitors. Atorvastatin, however, differs significantly from simvastatin (or lovastatin) in both structure and pharmacology because it is a “synthetic” and not a “natural” statin and does not cross the blood-brain barrier. Hence, direct parenchymal neuroprotective effects of atorvastatin as mechanism of stroke reduction seem unlikely. Only after the onset of cerebral ischemia may atorvastatin enter the brain parenchyma because of blood-brain barrier breakdown. In conclusion, our data indicate that the neuroprotective mechanisms of atorvastatin are predominantly mediated by NO-dependent effects of statins on hemostasis and blood flow.

The mechanism by which HMG-CoA reductase inhibitors increase eNOS expression is the inhibition of geranylgeranylpyrophosphate (GGPP), an isoprenoid intermediate of the cholesterol synthesis pathway. GGPP is important for the posttranslational modification of the small GTP-binding protein Rho, and Rho negatively regulates eNOS mRNA stability. In this study we demonstrate that atorvastatin treatment significantly inhibits Rho activity in the vessel wall in vivo. Therefore, in vitro and in vivo evidence suggests that statins upregulate endothelial NO production by inhibition of Rho isoprenylation independent of cholesterol synthesis.

Both type III (endothelial) and type II (inducible) NOS have been identified in human platelets and megakaryocytic cells. Platelet-derived type III NOS has been shown to regulate platelet function. Accordingly, incubation of platelets with the NOS substrate L-arginine inhibits platelet aggregation, whereas the NOS inhibitor L-N-monomethyl-L-arginine enhances platelet reactivity. NO release from activated platelets markedly inhibits platelet recruitment and may thus limit the progression of intra-arterial thrombosis.

Although the role of endothelium-derived NO has been extensively characterized, relatively little is known about the regulation of platelet-derived NO. In this study we show that pharmacological intervention, ie, inhibition of the mevalonate pathway by HMG-CoA reductase inhibitors, upregulates type III NOS expression in platelets in vivo. The associated decrease of 2 markers of platelet activation, PF 4 and β-TG, and the fact that thrombus formation was inhibited demonstrate the functional relevance of this observation. Platelet reactivity was indeed regulated by eNOS because PF 4 and β-TG plasma levels were not affected by statin treatment in eNOS knockout mice. Thus, we identify platelet-derived NO as a novel target for drug interventions.

Thrombosis superimposed on atherosclerosis is a key event in the pathogenesis of cerebral infarctions. The use of antiplatelet drugs is a well-established therapy for the secondary prevention of stroke. Recent evidence points toward the importance of platelet aggregation not only during cerebral ischemia but also as the acute precipitating event in most acute coronary syndromes. The development of acute coronary syndromes is attributed to thrombus formation on a fissured, eroded, or ruptured plaque in the coronary artery. Indeed, platelet activation is increased in patients with unstable angina pectoris. Therefore, patients at risk for both stroke and myocardial infarction significantly benefit from inhibition of platelet aggregation.

Hypercholesterolemia has recently been linked to platelet function because it facilitates platelet aggregation. Moreover, several recent studies have demonstrated beneficial effects of statin therapy on platelet function and fibrinolysis in hypercholesterolemic individuals. Our findings, by contrast, demonstrate that statins inhibit platelet activation independent of serum cholesterol levels by upregulation of type III NOS. Therefore, our data may have direct clinical implications: eNOS upregulation may be the mechanism of protection in patients with average cholesterol levels, as observed in large statin trials.
fact, we suggest that the decreased incidence of cerebrovascular events observed in these trials may in part be due to a reduction in cerebral infarct size to levels that are clinically unappreciated. In conclusion, our findings suggest that patients at risk for stroke or acute coronary syndromes may benefit from statin treatment regardless of their serum cholesterol levels.

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Despite the established role of cholesterol in the pathogenesis of coronary artery disease, current epidemiological evidence does not demonstrate a clear relationship between the risk of stroke and serum cholesterol level. However, recent studies indicate that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, or “statins,” significantly reduce ischemic stroke. The mechanisms underlying this reduction have not been fully elucidated and likely include the effects of statins on atherosclerotic plaque development and stability in the aorta and carotid artery. Other putative effects may include beneficial actions on cerebral endothelial function, cerebral hemorheology, and effects on platelet activation and thrombosis.

Perhaps most fascinating is the emerging body of data demonstrating effects of statins that occur independent of changes in cholesterol level. Many of these effects are thought to result from the depletion of isoprenoids. Isoprenoids are derivatives of intermediates in cholesterol biosynthesis and have a number of actions, including effects on G-proteins, adhesion molecules, and cell proliferation and signaling. Some of the cellular effects of statins, including effects on nitric oxide, may be mediated by reduced isoprenoid bioavailability.

NO produced by endothelial NOS (eNOS) has a protective physiological role and orchestrates the paracrine homeostatic functions of the endothelium, which include inhibition of leukocyte and platelet adhesion, control of vascular tone, and maintenance of a thromboresistant interface between the bloodstream and the vessel wall. Consistent with the concept that eNOS plays a protective role in focal cerebral ischemia is the observation that eNOS knockout animals experience larger infarcts after middle cerebral artery occlusion. In a murine model of ischemic stroke, prophylactic statin therapy with both simvastatin and lovastatin augments cerebral blood flow, reduces infarct size (by approximately 30%), and improves neurological outcome in normocholesterolemic animals. This study demonstrated that statin therapy directly up-regulates eNOS activity in the brain without altering expression of neuronal NOS. These effects occurred independent of changes in cholesterol level and were reversible by cotreatment with mevalonate or geranylgeranylpaphosphate.

The preceding study corroborates these findings with use of a different statin and adds to our understanding of possible mechanisms through which statins may be protective in cerebral ischemia. This study demonstrates that atorvastatin significantly reduces stroke size in normocholesterolemic mice independent of effects on cholesterol level. This effect on ischemic stroke appears to be mediated by upregulation of eNOS in the vasculature and platelets and through decreased platelet activation. The effect on platelet eNOS is particularly interesting and suggests that the putative antithrombotic effects of statins are not exclusively due to modulation of the endothelial eNOS system. These experiments also suggest that atorvastatin upregulates vascular and platelet eNOS by reducing the isoprenylation (and hence activity) of the small GTP-binding protein rho, which itself may negatively regulate eNOS mRNA. An evolving paradigm is that of cell function orchestrated by isoprenoid metabolites derived from within the cholesterol biosynthetic pathway. This is important because it may, to some degree, explain the unresolved disparity between epidemiological studies and clinical trials of cholesterol and stroke. The emerging data from both the bedside and the bench underscore the necessity for further clinical studies to explore the impact of statin therapy in human stroke and neuroprotection.

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